Development of a Vaccine for Bacterial Kidney Disease in Salmon

Annual Report FY 1985

by

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EXECUTIVE SUMMARY

Bacterial kidney disease (BRD) has been and remains a chronic contributory problem limiting the productivity of salmon in the Columbia River Basin. Control of this disease will not come easily, but it would lead to a tremendous increase in the health and numbers of our salmon populations. Vaccination of salmon to Renibacterium salmoninarum (KDB) is a potentially successful method of controlling this disease. To date, however, no successful vaccine has been developed for general use. A possible solution to this problem, and thus the goal of this research, is to isolate the antigenic components of KDB and enhance their ability to activate the host defenses. This will be accomplished by the chemical modification of these antigens with potent immunomodulatory substances. These modified antigens will then be tested for their effectiveness in inducing immunity to BKD and thereby preventing the disease.

The goal of the project's second year was to chemically modify the major antigens of Renibacteirium salmoninarum, immunize coho salmon (Oncorhynchus kisutch), and to test the immunogenicity of the preparations used. Immunogenicity of the antigenic material was tested by 1) admixture experiments, using whole KD cells with muramyl dipepetide, Vibrio anguillarum extract, E. coli lipopolysaccharide, or Mycobacterium tuberculosis in Freund's complete adjuvant.

In addition to these goals a number of important techniques have been developed in order to facilitate the production of the vaccine. These procedures include: 1) the use of the soluble antigen for diagnosis in the ELISA and Western blot analysis, 2) detection of salmonid anti-KD antibodies

by an ELISA technique, 3) detection of cellular immune responses to the soluble antigen, and 4) development of immersion challenge procedures for bacterial kidney disease (BKD).

TABLE OF CONTENTS

INTRODUCTION

Geographical Distribution

Bacterial kidney disease (BKD) is one of the most widespread diseases in the Columbia River Basin and occurs in many parts of the United States, Canada, Europe (Fryer and Sanders, 1981) and Japan (Kimura and Awakura, 1977). The first reported identification of what was most likely *R. salmoninarum* or kidney disease bacterium (KDB), was in 1930. Smith (1964) communicates that gram positive diplococci were isolated from Atlantic salmon (*Salmo salar*) found in Aberdeenshire in the River Spey in Scotland. The first recorded cases in the United States occurred in hatcheries in the state of Massachusetts as described by Belding and Merrill (1935). Not only is this disease a very grave problem for hatchery reared salmonids, but it has also been demonstrated to occur in wild populations (Evelyn, et al., 1973).

The Pathogen

The kidney disease bacterium (KDB) is described as a non-motile, non-spore forming diplobacillus. Original attempts to isolate the organism failed due to its rather fastidious requirements for growth in culture media. The first successful attempts at cultivation utilized media with such rich supplements as beef serum and fish extract (Earp et al., 1953). Later work of Ordal and Earp (1956) made use of cysteine blood agar, which contained 20% human blood as well as 0.1% cysteine-HCl. The most recent modifications obviates the need for blood

or serum, thus reducing the cost. This medium employs 0.1% L-cysteine-HCl in Mueller Hinton medium (Wolf and Dunbar, 1954).

Superficially the organisms, especially when seen within the tissues, resemble corynebacteria, thus they have been referred to as corynebacteria and the disease as corynebacterial kidney disease (Ordal and Earp, 1956; Hunn, 1964; Wedemeyer and Rose, 1973). Certain aspects of the pathology caused by this organism resemble not only corynebacterial disease, but to some degree diseases caused by mycobacteria and listeria. However, these rather circumstantial methods of characterization were not satisfactory for rigorous taxonomic classification. Fryer and Sanders (1980) explored this taxonomic problem on the molecular level, and determined that in regards to the guanosine/cytosine (GC) content, peptidoglycan and cell wall composition, these organisms were quite unique. Due to these singular molecular characteristics, these organisms have been placed in their own genus and species, Renibacterium salmoninarum. Isolates from various regions of the world also seem to share only a single serotype (Getchell, 1983).

Disease Pathology

The disease caused by KDB is considered to be a chronic systemic disease, with lesions occurring through much of the viscera and musculature in advanced cases. A common route of entry for the organism has not been demonstrated, and it seems plausible that infection may occur by various routes. Wood and Wallis (1955) have demonstrated that the ingestion of infected salmon flesh by chinook salmon leads to a lethal infection. Alternatively, eye trauma has also been suggested as a possible route of infection. Hendricks and

Leek (1975) found that chinook salmon possessing exophthalmia, demonstrated granulomatous lesions behind the affected eye which contained large numbers of leukocytes and KDB. Many of the fish which possessed this exophthalmia, had no other lesions internally. However, if all fish which exhibited this exophthalmia were held in aquaria, it was found that they all succumbed to BKD within two to three months. Upon necropsy, the animals were found to have disseminated BKD lesions. It was felt that the eye may serve a primary route of infection in hatchery-reared salmonids, due to the frequency of eye trauma when the animals are maintained in raceways. A similar, but perhaps a more common route of infection, may be through general abrasion of the body surface (Wolf and Dunbar, 1959).

Although there are many organs which become infiltrated with KDB, most investigators feel that the primary target is the kidney. The hematopoietic portion of the anterior kidney appears to be especially susceptible. It is a diagnostic feature of the disease to see white granulomatous areas of infection within the kidney. When examined microscopically these areas are seen to possess KD organisms. These foci of infection are not limited to the kidney, but they are found to appear also in the spleen and liver. As the disease progresses, the reproductive organs, musculature, and brain often become infected. Externally, besides the exophthalmia, pustules or blebs may be seen above the lateral line and petechial hemorrhaging around the muscles of the peritoneum.

One of the most striking features of the internal pathology of BKD is the development of a white pseudomembrane composed of dead host tissue, bacteria, and leukocytes. This false membrane has been seen to cover the liver, reproductive organs, spleen, and

occasionally the swim bladder (Snieszko and Griffin, 1955). This phenomenon is not unlike the pseudomembrane produced in diptheria infections in man. The formation of this pseudomembrane, however, is quite temperature dependent. It is reported that it forms at temperatures below 8.3°C, whereas at higher temperatures only necrosis is found (Smith, 1964). Attempts to isolate an exotoxin as is expresssed by Corynebacteria diptheriae have not met with success.

On the cellular level, bacterial kidney disease appears to be more similar to listerial or mycobacterial infections. A common feature of these diseases are the granulomatous reactions that occur. Like listeria and mycobacteria, KDB organisms are phagocytized by macrophages, but are not always digested by the phagocytic cells (Young and Chapman, 1978). In fact, the KDB as well as listeria and mycobacteria have been observed to multiply within the macrophage itself. In the case of mycobacteria, the cellular arm of the immune response is eventually activated to destroy the bacteria. The usual delay in this response, coupled with physiological mechanisms the pathogen uses to subvert the phagocytic response leads to a widespread infection. With regards to a specific antibody response, intracellular organisms such as KDB are thought to be relatively resistant, while within their host cell. In this situation, although the pathogen may be protected, the immune system of the host is still exposed to a continuous supply of antigen from the pathogens. As a result there is a continuous severe immune reaction which eventually destroys the surrounding host tissue in attempting to destroy the pathogen. This immune reaction leads to much necrosis and characteristically severe granulomatous lesions. Since KDB is harbored by macrophages of the fish and these severe granulomatous reactions occur in response to the antigen of the pathogen, it is not surprising that two of the organs that are most severely affected are the spleen and kidney. Both

of these organs are immune organs and contain a great number of macrophages.

Effect on the Kidney

It is felt that terminal cases of BKD may be fatal due to the destruction of the kidney which, in turn, may lead to an inability of the salmon to osmoregulate (Frantsi et al., 1975). This feature of BKD is extremely important in light of the evidence that salmon infected with BKD demonstrate marked increase in mortality when held in salt water as compared being held in fresh water (Banner et al., 1983). The possibility arises that even if fish seem relatively healthy or have recovered from BKD after antibiotic treatment, they may be at high risk once they enter the ocean, due to extensive kidney damage.

Kidney pathology, in salmon with BKD,looks quite similar to the pathology seen in glomerulonephritis in mammals. Glomerulonephritis could be mediated by either of two mechanisms: 1) immune complex formation beween KDB antigens and anti-KDB antibodies or, 2) by a reaction of anti-KDB with crossreactive kidney antigen on the basement membrane of the glomerulus. The precise mechanism by which this condition is elicited has yet to be discovered, however, due to the degree of bacterial infiltration that occurs throughout the body of the fish, it would seem most likely that an immune complex reaction could be occurring.

Tolerance

It seems obvious that in most cases of BKD the salmon are responding to the pathogen, but their response seems inappropriate and incapable of overcoming the pathogenic insult. Immunological tolerance may

play a role in BKD if it serves to delay or produce an inappropriate immune response.

If a foreign antigen is present within the body early in life, the animal may experience a state of immunological tolerance (Billingham et al., 1953). In this state the animal does not recognize that particular foreign antigen as being different from its own body and; therefore, it will not respond to it immunologically. This phenomenon may apply in some cases to BKD. It has been demonstrated by Evelyn et al. (1984) that eggs from females with BKD possess the pathogen within the yolk. This was demonstrated microscopically and by iodine treatment of the egg surface. The iodine treatment should have killed any pathogen on the surface of the egg or residing within the perivitelline space, but the organism persisted. If this evidence is correct, it would support the hypothesis of vertically transmitted of BKD and would indicate a route by which fry might experience conditions similar to that giving rise to neonatal tolerance in mammals.

Induction of Protective Immunity

The appearance of circulating antibody does not correlate with protection from the disease. Although this intracellualr location of the pathogen may seem inaccessible, it is not. Mycobacteria and listeria also reside within phagocytes and these pathogens can be controlled if the host is properly sensitized or immunized. Listeria, which possesses physical characteristics similar to those of KDB (Bullock et al., 1975), appears to be insensitive to specific antibody. Priming of the cell mediated immune response, however, results in the elimination of these pathogens. Induction of the cell mediated (T cell) response results in the activation of the phagocytic cell and digestion of the bacteria residing there (Mackaness, 1969).

It is possible to enhance this cell mediated immune rsponse, as well as the immune response in general though the use of adjuvants. Adjuvants utilizing bacteria such as mycobacteria or corynebacteria lead to an enhancement of the immune response to the admixed antigen. Such augmentation may aid in the control of BKD. It has been reported that intraperotineal injections of KDB emuldified in oil and mycobacteria (Freund's complete adjuvant) can lead to a reduction of BKD lesions and organisms (Paterson et al., 1981). Although such immunization procedures would be impractical for large scale vaccine programs, they do demonstrate that proper presentation of KDB antigen to the fish can lead to a protective state of immunity.

MATERIALS AND METHODS

Animals. Fingerling-three year coho salmon (50-300 g) were kept in ambient (12°C) pathogen free well water, in 220 gallon circular tanks at the Oregon State University Fish Disease Laboratory (OSU-FDL). The fish were maintained on Oregon Moist Pellets. The disease challenges were performed on salmon housed at the OSU-FDL in 30-liter tanks, the effluent from which was chlorinated.

Adult female New Zealand White (NZW) rabbits and BALB/c mice were maintained by the Laboratory Animal Resource Center at Oregon State University.

Bacterial strain. R. salmoninarum isolate Lea-l-74 (ATCC 33209) obtained from J. R. Rohovec, Oregon State University, Corvallis, Oregon, was used throughout the study.

Growth conditions. Bacteria were grown in either unfiltered Kidney Disease Medium-2 (KDM-2) or ultrafiltered KDM-2 (UF-KDM-2). Both media were modified by the elimination of the bovine serum supplementation that was originally specified by Evelyn (1977). The UF-KDM-2 was prepared by passage of KDM-2 through a PTGC-10,000 NMWL filter packet in a Minitan ultrafiltration apparatus (Millipore Corp., Bedford, MA). This filtration produced media free of molecules with molecular weights greater than 10,000. Cultures were incubated for one to three weeks in low form culture flasks with constant agitation. At the end of the incubation period, the bacterial cells were centrifuged at 6000 x g for 30 minutes (4°C) and the supernatant fluid was saved for soluble antigen extraction.

Soluble antigen extraction. Culture supernatants were filtered,

as described above. The retentates, or high molecular weight fractions, were concentrated by 50% saturated ammonium sulfate (SAS) precipitation. After addition of the ammonium sulfate, the solutions were stirred for 3-4 hours at 4°C. The precipitate was removed by centrifugation at 6000 x g for 15 minutes (4°C) and suspended in l0-20 ml of 0.01 M phosphate buffered saline, pH 7.2 (PBS). The solution was reprecipitated twice and the resuspended precipitate dialysed extensively against PBS. The dialysed protein extract was assayed for protein by the method of Lowry et al. (1951). This process is graphically depicted in figure 1.

Vibrio anguillarum extract. The Vibrio extract was prepared from V. anguillarum strain SL-174 which had been formalin killed and stored frozen. Fifty mls thawed packed cells were suspended in 10 volumes of 2% saline and placed in a boiling water bath for two hours. Cells were washed three times in 2% saline, centrifuged at 10,000 x g for ten min at 4'C, resuspended in 95% ethanol, and incubated 48 hours at 37°C. The cells were then washed two times in acetone, centrifuging at 3,000 x g for 10 minutes and dried to a paste overnight at 37°C. The paste was ground to a fine powder with mortar and pestle and stored at 4°C. The soluble Vibrio extract used for these studies was prepared by boiling the powder in PBS at ten mg/ml in a boiling water bath for one hour with frequent agitation. This suspension was then centrifuged at 1,000 x g to remove particulates and filter sterilized. Protein concentrations were determined by the method of Lowry et al. (1951).

Other antigens: Trinitrophenylated-LPS (TNP-LPS) was prepared by the method of Jacobs and Morrison (1975). All antigens were diluted into tissue culture medium at two times the final desired concentration.

SOLUBLE ANTIGEN PREPARATION

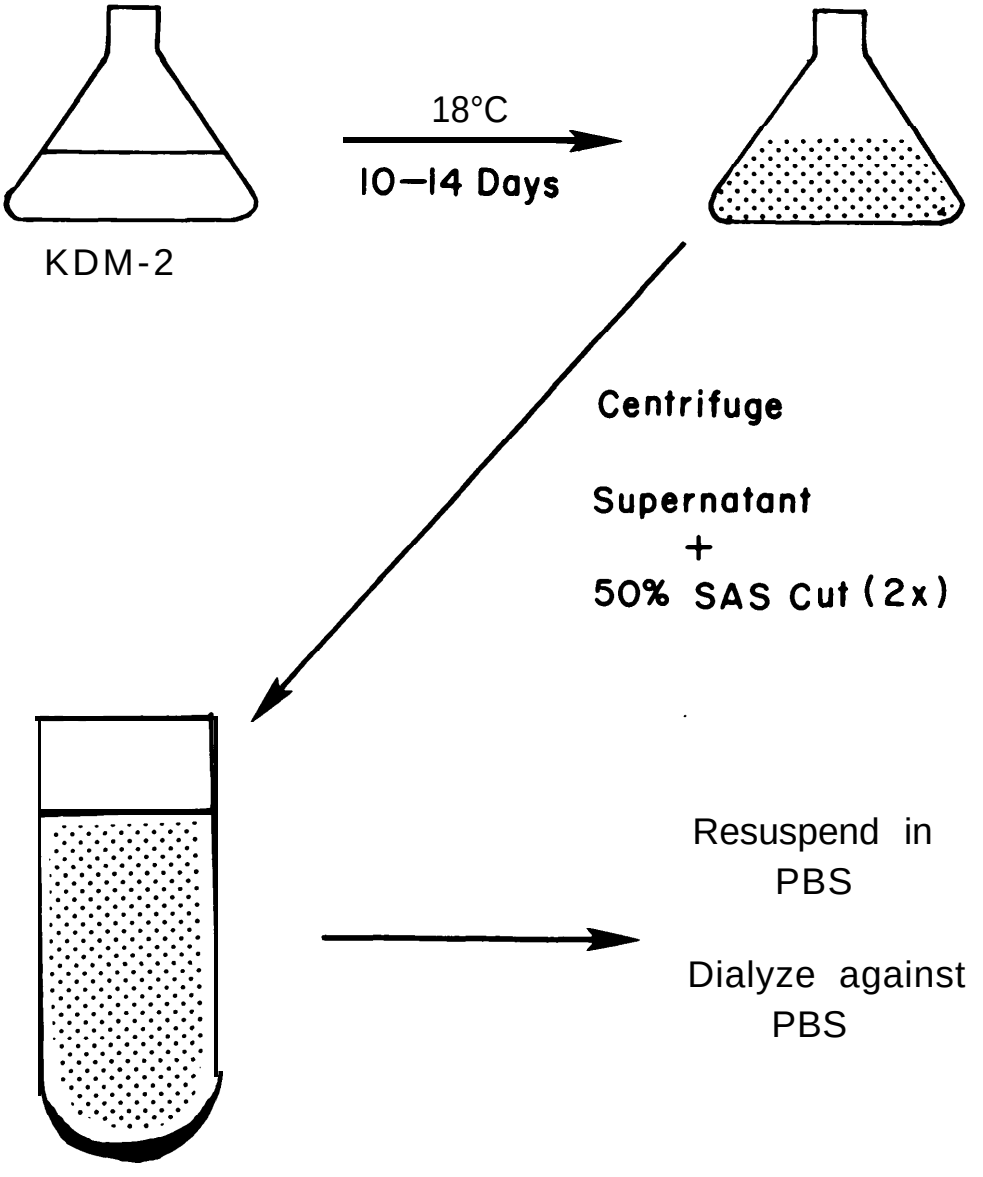


Figure 1. Flow diagram depicting the generation of soluble antigen.

Sonication. Sonicates of bacterial cells were prepared as described by Getchell (1983). Briefly, a suspension of 10% washed cells in PBS were exposed to four bursts at 50 watts from a Sonifier Cell Disruptor (Heat Systems Ultrasonics, Plainview,NY). Particulates were removed by centrifugation at 5000 x g and the supernate filter sterilized and stored at 4°C until used.

Antibody preparation. Female New Zealand white rabbits were injected subcutaneously between the scapulae (1.4 ml) and in the footpads (0.4 ml each) with a 1:l emulsion of immunogen and Freund's complete adjuvant (FCA). The rabbits werte rested 30 days then bled weekly. Seven weeks post-immunization the rabbits were boosted using the same protocol. The serum was aliquoted and stored at -70°C.

Immunogens used were formalin-killed R. salmoninarum, washed three times in PBS and resuspended to 1.0 OD_{520}; dialysed protein extract from UP-KDM-2 (1.5 mg/ml); and dialysed protein extract from KDM-2 (2.2 mg/ml).

Antisera to R. salmoninarum isolate K50 was kindly donated by J. R. Rohovec, O.S.U., Corvalli, Oregon.

Antisera from coho salmon were produced by monthly injections of 0.1 ml of a 1:l mixture of formalin-killed R. salmoninarum in FCA. Serum was aliquoted and stored at -70°C until used.

Gel filtration. Standard solutions of all antigen preparations were examined with respect to their molecular weight by the use of gel filtration. Briefly, each antigen preparation was eluted through a G-200 column (1.6 x 40 cm). Each sample was adjusted to a concentration of 2.2 mg in 500 ul. Elution was conducted using PBS as the elution buffer. The fractions were monitored for protein using the Lowry method. Molecular

weight standards used for the calibration of the column consisted of human IgG (150 kd), bovine serum albumin (68 kd), and cytochrome C (13 kd).

Polyacrylamidegel electrophoresis (PAGE). The method followed is basically that of Laemmli (1970). The samples were mixed with the sample buffer at a 1:l ratio and subjected to the electrophoretic field. The samples are usually loaded in a duplicate fashion so that one half is subjected to silver staining and the other half is used for Western blotting. During the electrophoretic run, a constant 20 mA current is applied in the stacking gel and a constant 40mA during the run when the samples are in the separating gel. After completion of the experiment the gel is cut into two halves and one half is silver stained by the method of Wray et al.( )

Western blotting. The procedure involves a modified methods of of Towbin et. al. (1979). After transfering the protein bands onto nitrocellulose paper, they are stained by the following method: 1) Soak electrophoretic blots in 3% bovine serum albumin in Tris buffered saline (pH 7.4) for one hour at 40^{o} C, 2) Rinse five times with TBS (30 minutes), 3) incubate with rabbit anti-KDB - HRPO conjugate for two hours at room temperature, 4) wash extensively with TBS, 5) put the nitrocellulose paper in the substrate solution (Bio-Rad,Richmond,CA). The reaction is then allowed to proceed at 37^{o} C until the protein bands stain darkly.

Isoelectric focusing. Isoelectric focusing was performed in a modified procedure of Jackson et al. (1980). Polyacrylamide gels (5%; 115 mm x 230 mm x 1 mm) were cast between glass plates and allowed to polymerize overnight. A pH gradient of 4-6.5 or 3-10 with a 5% carrier ampholyte (Pharmacia, Piscataway, NJ) concentration was used. The distance between the electrode wicks was 9.0 cm. The anolyte and catholyte were 0.04 M glutamic acid and 0.2 M (L)-histidine,

respectively. Samples were applied near the cathode by the use of applicator masks (Pharmacia, Uppsala, Sweden). The running conditions were a constant power of 30 watts for 30 minutes. The gels were silver stained according to the method of Merrill (1981).

Conjugation of horseradish peroxidase (HRPO) to antibody. Four mg of horseradish peroxidase (Sigma, St. Louis,MO) were dissolved in one ml of distilled water. To this solution, 200 ul of a 0.1 M solution of sodium meta-periodate (Sigma) in water was added and stirred at room temperature for 20 minutes. This mixture was then dialysed against 1 mM acetate buffer (pH 4.4) overnight at $4^{o}C$.

Simultaneously, a 50% saturated ammonium sulfate (SAS) globulin precipitate of 1 ml rabbit antiserum was reconstituted in 1 ml of distilled water and dialysed overnight at $12^{o}C$ in 0.01 M Na_2CO_3 pH 9.47, Twenty ul of this bicarbonate buffer was then added to the HRPO to raise it to a pH of 9.0-9.5. The rabbit globulins were then immediately added to the solution and stirred at room temperature for two hours. This conjugate was then dialysed against 0.01 M PBS with a dialysis memebrane having a pore size that included molecular weight species of greater than 50000. The conjugate was stored in the dark in 0.001% merthiolate.

ELISA for the detection of soluble antigens. A method for the detection of soluble KDB antigen was developed utilizing a capture antibody technique. Briefly, individual wells of Costar EIA l/2 well plate (Cambridge,MA) were coated with rabbit anti-soluble antigen (from either UF-KDM-2 or KDM-2. Antigen coating was accomplished by incubating the rabbit antibody diluted in carbonate buffer (0.05 M, pH 9.5) overnight at $4^{o}C$. The precise concentration of antibody varied depending upon the experimental protocol. Prior to use, the plates were washed three times in

a Tris/Tween diluent buffer (see appendix), followed by three washes in Tris buffer. Solutions containing soluble antigens or unknowns were diluted in Tris/Tween the added to the wells of the plate in 100 ul aliquots and incubated for two hours at room temperature. Following this incubation, the wash sequence was repeated. At this point 100 ul of rabbit anti-soluble antigen-HRPO was added to each well and incubated for two hours at room temperature. This incubation was followed by another standard wash and the addition of 100 ul of the sustrate solution (see appendix). Elaboration of a colored product was read spectrophotometrically at 405 nM by a Biotek Automatic ELISA Reader (Burlington,VT). Absorbance readings were proportional to the amount of soluble antigen bound to the plate since the conjugate was incubated in excess.

ELISA for antibody to KDB soluble antigen. This assay was basically prepared in a similar manner to the ELISA previously described (see figure 2). Briefly, 100 ul aliquots of soluble antigens were diluted in carbonate buffer and coated on the wells of an ELISA plate. After an overnight incubation at $4^{o}C$ the plate was washed and a 100 ul aliquot of a test serum dilution or hybridoma supernatant was incubated in the wells for two hours at room temperature. Following this incubation the plates were washed, and 100 ul of the appropriate dilution of rabbit anti-mouse IgGHRPO (Hyclone, Logan, Utah) was incubated on the plate for two hours at room temperature. After this incubation, the plates were washed and 100 ul of the substrate solution was added and read spectrophotometrically at 405 nM.

ELISA for antibody to KDB cellular antigens. This assay followed the identical procedures as described in the preceding section, except for the method of coating the antigen onto the plate.

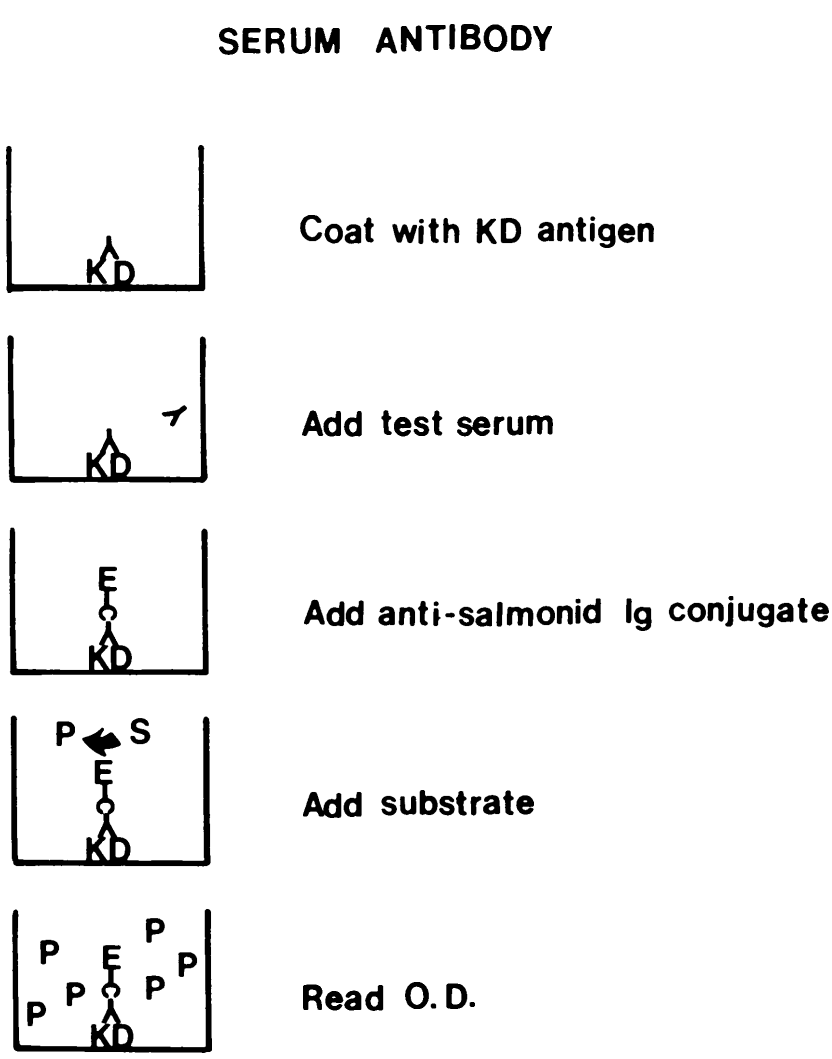


Figure 2. Flow diagram depicting the ELISA used in detecting salmonid anti-BKD antibodies

The antigen in this procedure is the whole bacterial cell and thus it does not readily adsorb to the ELISA plate. To facilitate binding of the whole KDB cell, the wells must first be coated with 100 ul of a 0.1 mg/ml solution of poly-L-lysine in water. The poly-L-lysine is allowed to adsorb for two hours at room temperature. Wells were then washed once in PBS and then incubated with dilutions of KDB in PBS. The cells were allowed to adsorb for one hour at room temperature. The wells were then washed twice in PBS. This wash was followed by an incubation of 100 ul of 0.25% glutaraldehyde for three minutes Following this incubation, the plates were washed three times with PBS and the remaining reactive sites were blocked with the addition of a solution of 10 mg/ml bovine serum albumin (BSA) in 100 mM glycine. Plates were blocked overnight at room temperature before use.

Monoclonal antibody production. Adult female BALB/c mice (Simonsen Laboratories, Gilroy, CA) were immunized with either 100 ug soluble antigen or 1 O.D. unit of formalin-treated KDB emulsified in Freund's complete adjuvant. Sera from immunized animals were tested at one month. Animals with positive titers, as determined by the previously described ELISA, were challenged intravenously (i.v.) with 10 ug of the same antigen in physiological saline. Animals with negative titers were boosted with the initial antigen preparation until a positive titer appeared.

Three days after i.v. challenge, single cell suspensions were prepared from the spleen and fused with a equivalent number of SP/2 myeloma cells by the aid of polyethylene glycol (Fisher, Fair Lawn, NJ). Hybridomas were selected by addition of hypoxanthine-thymidine -aminopterin (HAT) medium (Oi and Herzenberg, 1980). Productive fusions were ascertained by screening the hybridoma supernatants with the previously

described ELISA. Cells from these fusions were cloned and stored in liquid nitrogen until used.

Culture medium: All in vitro cultures employed the one medium described here. Media components were purchased from Whittaker M. A. Bioproducts, Walkersville, MD, unless otherwise noted. Mishell-Dutton holding medium (HM) consisted of 100 ug/ml gentamicin and 10% fetal calf serum in RPM1 1640 (Gibco). Mishell-Dutton modified RPMI (RPM1 MDM) was used for tissue culture and consisted of RPM1 supplemented with: non-essential amino acids, sodium pyruvate, L-glutamine, 10% fetal calf serum (hybridoma screened), 100 ug/ml gentamicin, 50 uM 2-mercaptoethanol (MCB, Cincinnati, OH), and the nucleosides, adenosine, uracil, cytosine, and guanine (10 ug/ml, Sigma, St. Louis, MO). The nutritional cocktail was also prepared as previously described (Tittle and Rittenberg, 1978) and fed daily to the cultures as described below.

Cell Cultures: Fish were sacrifice and their spleens and/or anterior kidneys were aseptically removed and placed in holding medium. A single cell suspension of each organ was obtained by aspiration through a one ml syringe. Organs from multiple fish were pooled to obtain the required number of cells for culture. The resulting cell suspension was incubated on ice to allow organ fragments to settle. The supernatant medium, containing a single cell suspension, was then washed two times in holding medium by centrifugation at 500 x g for 10 minutes at $4^{o}C$. The final washed cell pellet was resuspended in RPMI MDM. Lymphocytes were enumerated by the use of a hemocytometer or Coulter counter (Coulter Electronics, Hialeah, FL) adjusted for counting salmonid leukocytes. The cell suspension was then adjusted with RPMI MDM to a concentration of $2x10^{7}$ cells/ml and held on ice until culture. 0.2 ml aliquots of the final cell suspension were added to the wells of a 24-well, flat-bottomed, tissue culture plate

(Corning, Corning, NY) containing antigen or mitogen. Tissue culture plates were then incubated in plastic culture boxes (C.B.S. Scientific, Del Mar, CA) in an atmosphere of 7% CO_2 at 16°C. The cultures used for PFC assays were maintained by adding 25 ul of cocktail on alternate days.

Plaque-forming Cell Assay. Single cell suspensions of $2X10^7$ cells/ml were prepared in RPMI MDM, as described above. Aliquots of 0.2 ml were added to 0.2 ml of the appropriate dilution of antigen in RPMI MDM or in medium alone. Cultures were fed 50 ul of feeding cocktail on alternate days until harvest. Cells secreting anti-trinitrophenyl (TNP) or anti-soluble antigen antibodies were detected by a modification of the Cunningham plaque assay (Cunningham and Szenberg, 1968). 100 ul (40 ul for final assays) of the lymphocyte suspension, 25 ul (10 ul) of a 10% suspension of TNP-sheep red blood cells (TNP-SRBC; Rittenberg and Pratt, 1969) in modified barbital buffer (MBB), and 25 ul (10 ul) of steelhead serum, diluted in MBB, were mixed in individual wells of a 96-well microtiter plate (Linbro, McLean, VA). The contents of each well was pipetted into a slide chamber, sealed and incubated for l-2 hours at 16'C. Plaques were then enumerated under low power with the aid of a dissecting microscope. KD SRBC were produced by chromic chloride conjugation (Vyas et al., 1968).

Vibrio Assays. For the mitogen assays, 50 ul of the cell suspension ($1X10^6$ cells/ml) were placed in individual wells of a 96-well flat bottom tissue culture plate with 50 ul of mitogen or culture medium. The plates were then incubated in gas boxes under 7% CO_2 at 17°C. Twenty four hours before harvest each well was pulsed with 1 uCi of tritiated thymidine (methyl-^3H, ICN Biomedicals, Irvine, CA) in 50 ul of RPM1 MDM. Cells were harvested with distilled water onto glass fiber filters, with a Skatron cell harvester (Sterling, VA). The filters were then dried, placed in scintillation vials with cocktail (6g PPO, Sigma, 5 mg POPOP, Amersham,

Arlington Heights, IL, in 1 liter toluene, after Etlinger et al., 1976), and counted on a Beckman liquid scintillation counter (EL 3800). Data are reported as mean counts per minute (cpm) +/- standard error, of triplicate cultures, or as stimulation indices (SI) defined as experimental cpm/control cpm.

Glutaraldehyde con.jugation. This form of conjugation basically follows the procedures of Avrameas (1969). Briefly, the bacterial suspension is diluted with phosphate buffered saline (PBS - pH 7.2) to a concentration of $3x10^8$ cells/ml. To two-five ml of this suspension, one ml of KD soluble antigen (2 mg/ml) is added and mixed in the suspension. Twenty ul of 25 % glutaraldehyde (Sigma, St. Louis, MO) is added and the suspension is allowed to react for 2 hrs on ice. The preparation is dialysed overnight in PBS ar 4^o C. The suspension is then centrifuged at 6130 x g for 20 minutes and the supernatant removed. The cellular pellet resuspended to the original cellular concentration of $3x10^8$ cell/ml.

Bacterial agglutination. agglutinations were performed in 96-well round bottom microtiter plates. Briefly, 50 ul of PBS dileunt are deposited into all wells. 50 ul of the serumis then added to the first well of a row. A microdiluter is then used to perform the serial l/2 dilutions of the antiserum. Following these dilutions, 50 ul of the conjugate ($3x10^8$ cells/ml) is added to each well. The plate is then tapped to insure complete mixing, and the plate is examined for agglutination after an overnight incubation.

Conjugation with cyanuric chloride - Gray's (1979) method. Vibrio antigen extract (VAE) was used at a concentration of one mg/ml. 0.5 ml of this material was mixed with 1 ml of 0.01 N NaOH, containing 0.001% phenophthalein indicator. Cyanuric chloride was added in various amounts (0.4, 0.6, and 1.0 mg). After this solution was mixed for 10 seconds, the

solution was then added to 0.5 ml of a 0.5 or 1.0 O.D. suspension of KD cells. The cells were then centrifuged at 6130 x g for 30 miutes at 4^{o} C. The cellular pellet is then resuspended to its original volume of one ml.

Conjugation with cyanuric chloride - Scibienski's method. This method of conjugation is taken from that of Scibienski et. al. (1977). Briefly 0.5 mg of Vibrio extract is added to 1 ml of 0.15 M $NaHCO_3$ and kept constantly stirring while held on ice. Cyanuric chloride (0.2 ml of a 10 mg/ml) in acetone is added to the reaction mixture. The mixture is then stirred for 30 minutes on ice. KDB cells (2 ml of a 1.0 O.D. solution in $NaHCO_3$) are added to the solution. This mixture is then incubated for four hours at toom temperature, while stirring. the cells are centrifuged at 6130 x g for 30 minutes at 4^{o} C. The cells are resuspended to the original volume with PBS and dialysed in PBS overnight at 4^{o} C.

Vibrio extract preliminary characterization. A series of ammonium sulfate precipitations were done on one sample of Vibrio extract. Incremental volumes of saturated ammonium sulfate (SAS) were added to a sample of Vibrio extract suspended in PBS. After addition of the SAS solutions were stirred for two hours on ice and the precipitate removed. Each further addition of SAS yielded further precipitation. All precipitates were then resuspende to the original, uncut volume (2 ml) and dialysed against PBS. The final supernatant was also dialysed to remove the ammonium sulfate, and subsequently concentrated by sucrose, followed by another dialysis against PBS.

RESULTS AND DISCUSSION

Effects of immunostimulatory agents - admixture experiments.

In these studies, the effects of the various immunostimulatory agents (muramyl dipeptide, Freund's complete adjuvant, *E. coli* lipopolysaccharide, and *Vibrio anguillarum* extract) were examined without the covalent attachment to the antigen. Thus, although in a practical vaccine covalent linkage would be essential, administration of both components could be tested by mixing the agent with the antigen within the oil vehicle of Freund's incomplete adjuvant. Subsequent intraperitoneal injection would insure that the animal is simulataneously exposed to both the agent and the antigen. Thirty animals were injected for each group.

On day 35, five animals from each group were sacrificed for serum, anterior kidney, and spleen samples. The remaining animals were then challenged with KDB. Analysis of the serum samples by anti-KD ELISA, revealed no anti-KD antibodies. However the *in vitro* cellular responses did reveal some differences (Fig 3). As can be seen from the figure the groups immunized with KDB with muramyl dipepetide or *Vibrio anguillarum* extract demonstrate high levels of stimulation with anterior kidney cells and marginal stimulation with Freund's complete or lipopolysaccharide. Cells from control animals actually appear to be suppressed by the presence of the KD soluble antigen. In the spleen, only lymphocytes isolated from the group receiving *Vibrio* extract with the KDB appeared to give some response. It, thus, would appear for this data that muramyl dipepetide and *Vibrio* to be best able to stimulate a response. No stimulation was seen with FIA alone.

The final mortality data revealed no significant differences, between all treatment groups (Fig 4,5,6). In fact, the control (6F) had fewer mortalities

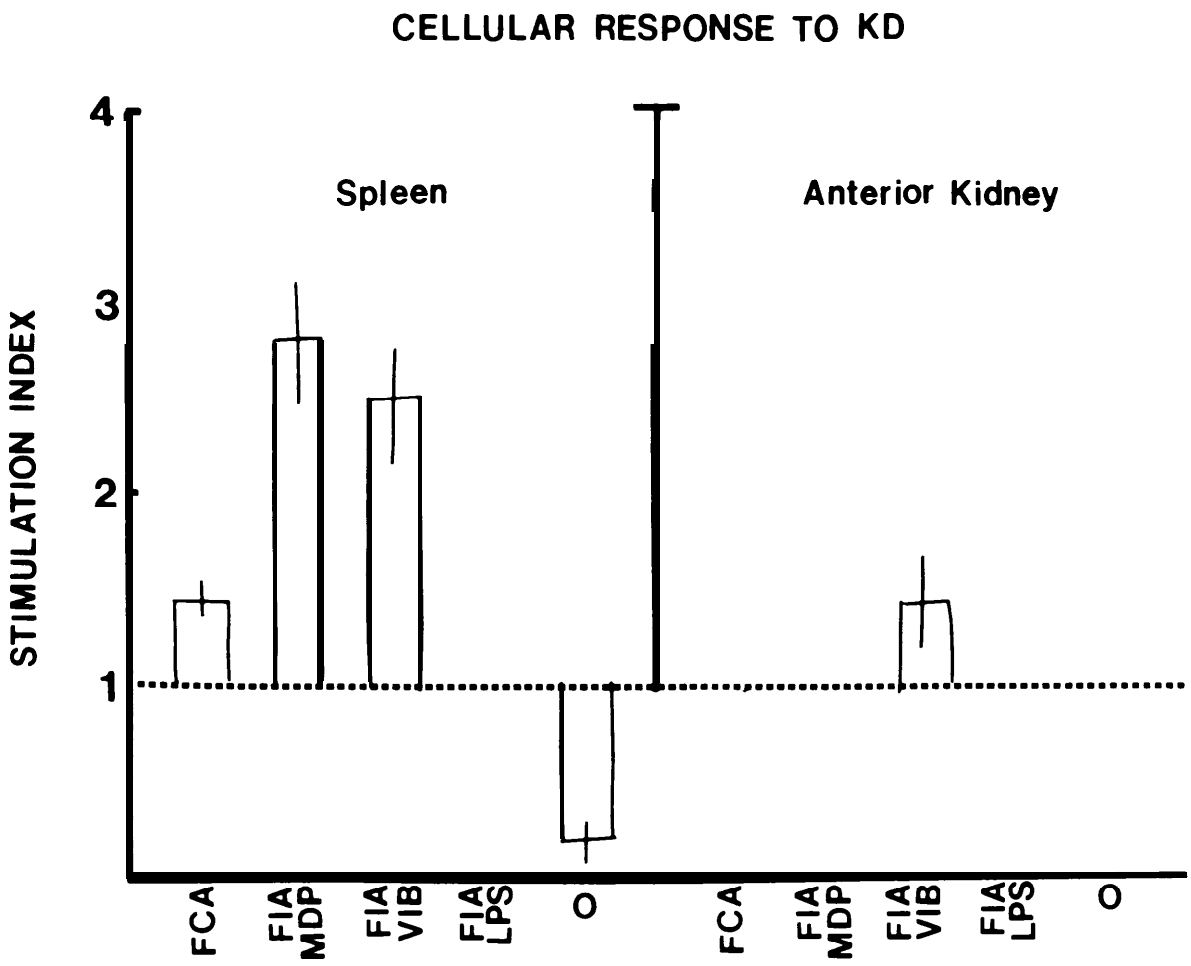
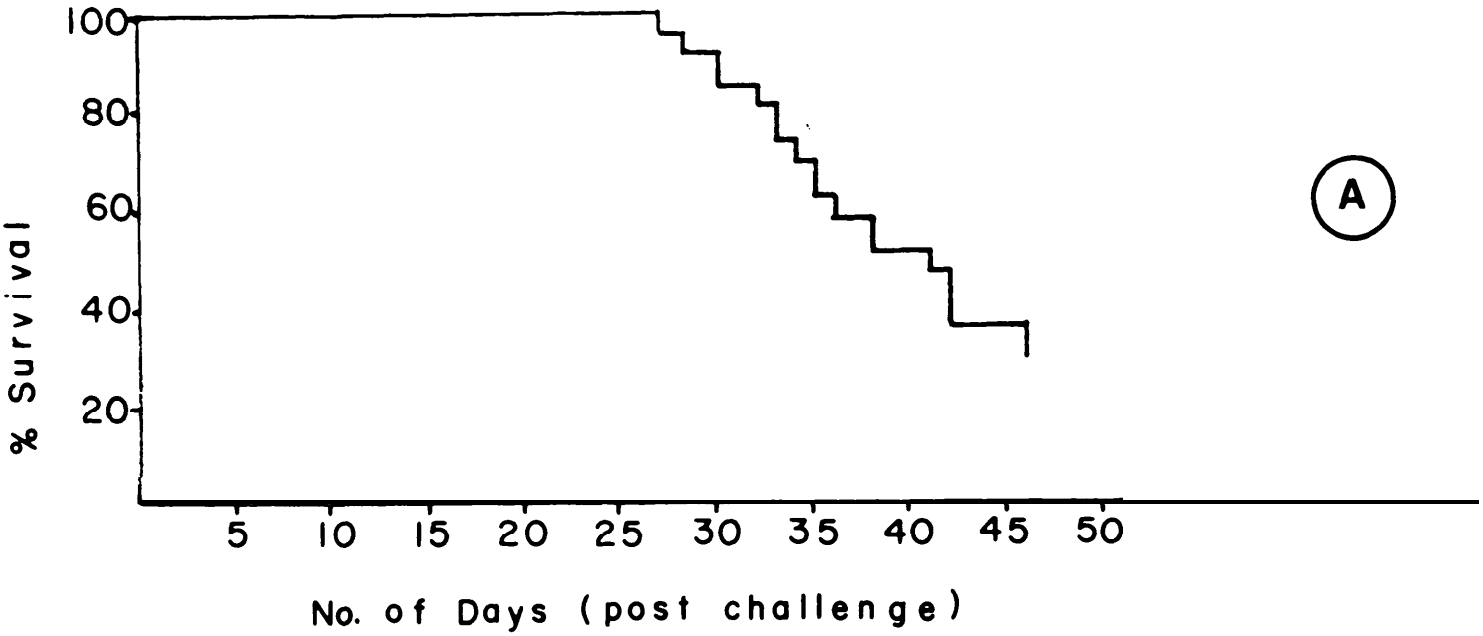


Figure 3. Cellular response to KD - Admixture experiment. Effect of immunization on the ability of spleen and anterior kidney lymphocytes to respond to soluble antigen in vitro. The error bars represent two standard errors about the mean of triplicate samples.



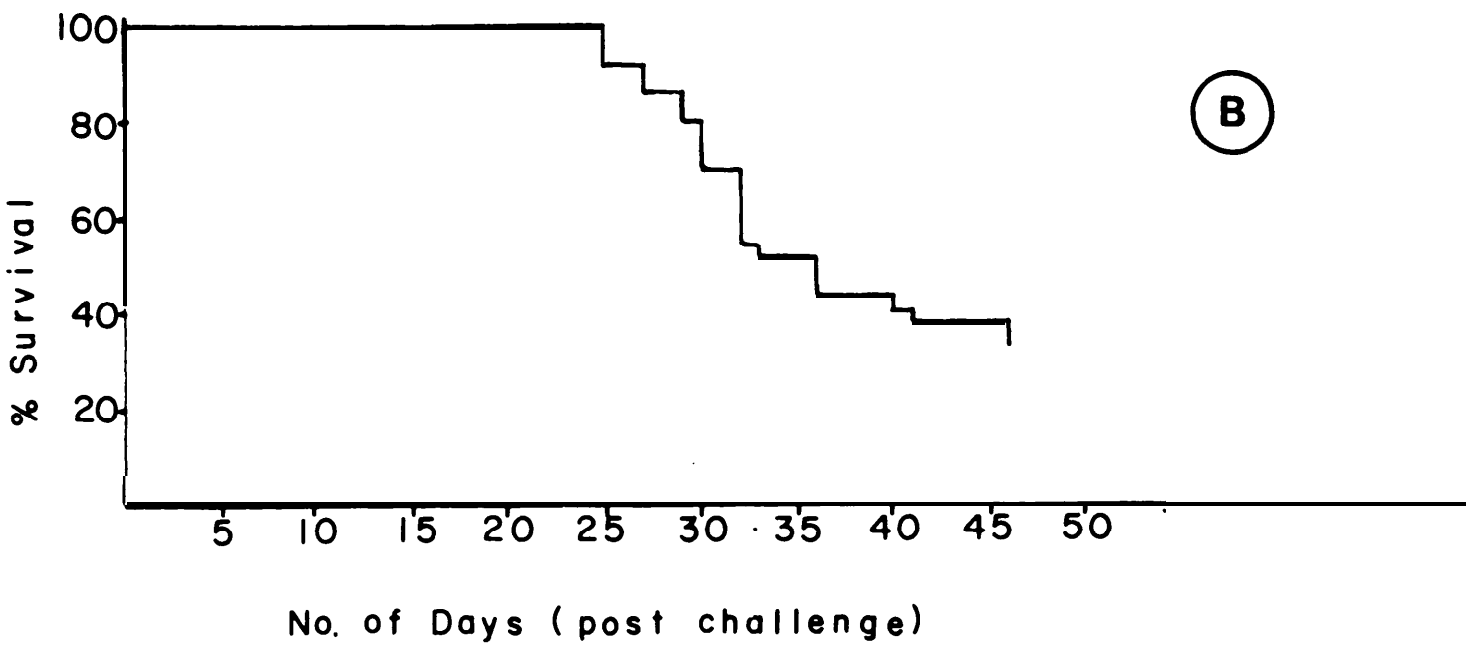


Figure 4. Cumulative mortalities - admixture experiment. Thirty animals were injected with 0.1 ml of a 1.0 O.D. KDB suspension. A = animals immunized with KD cells in the presence of MDP, B = animals immunized with KD cells in Freund's Complete adjuvant.

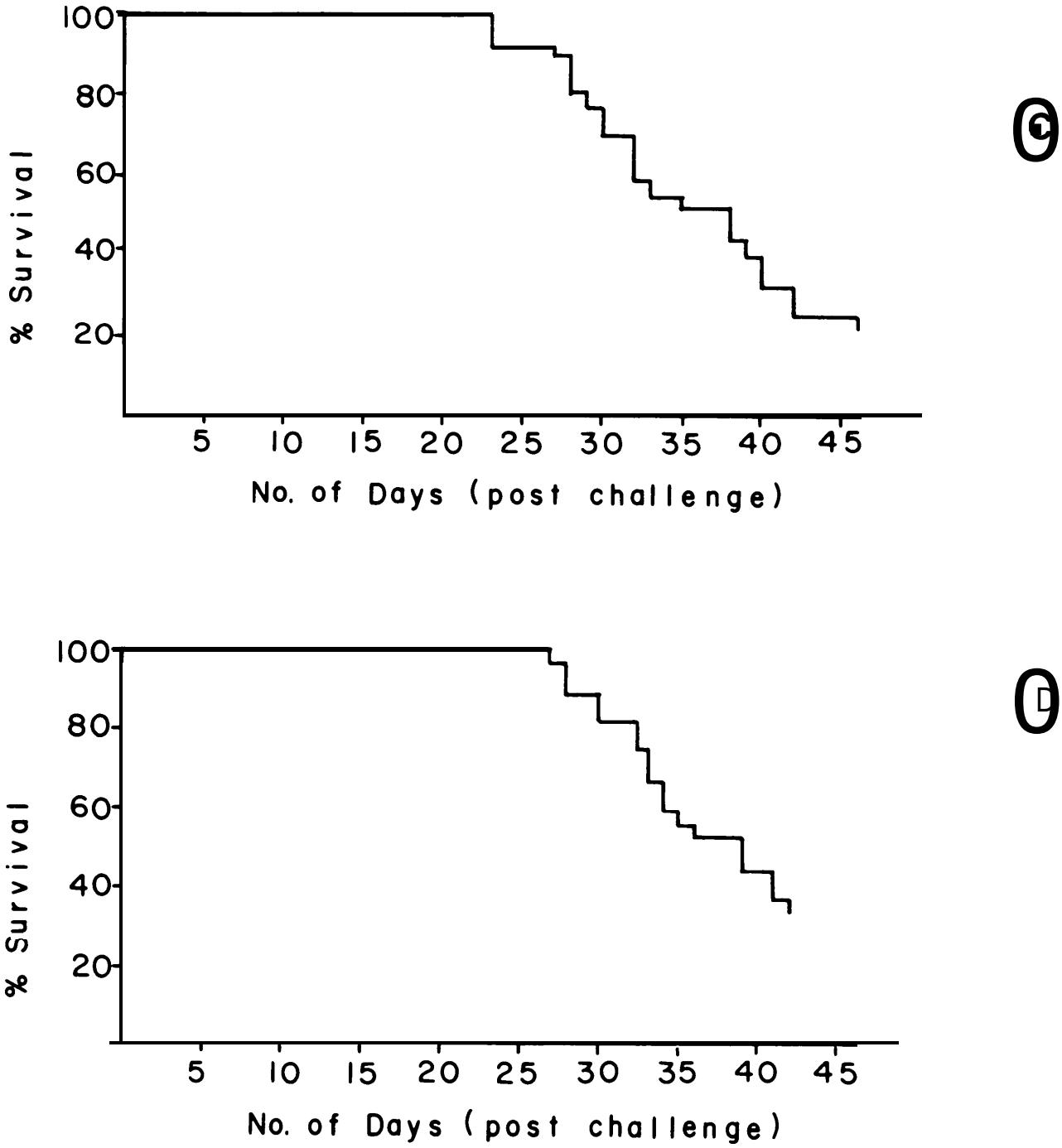
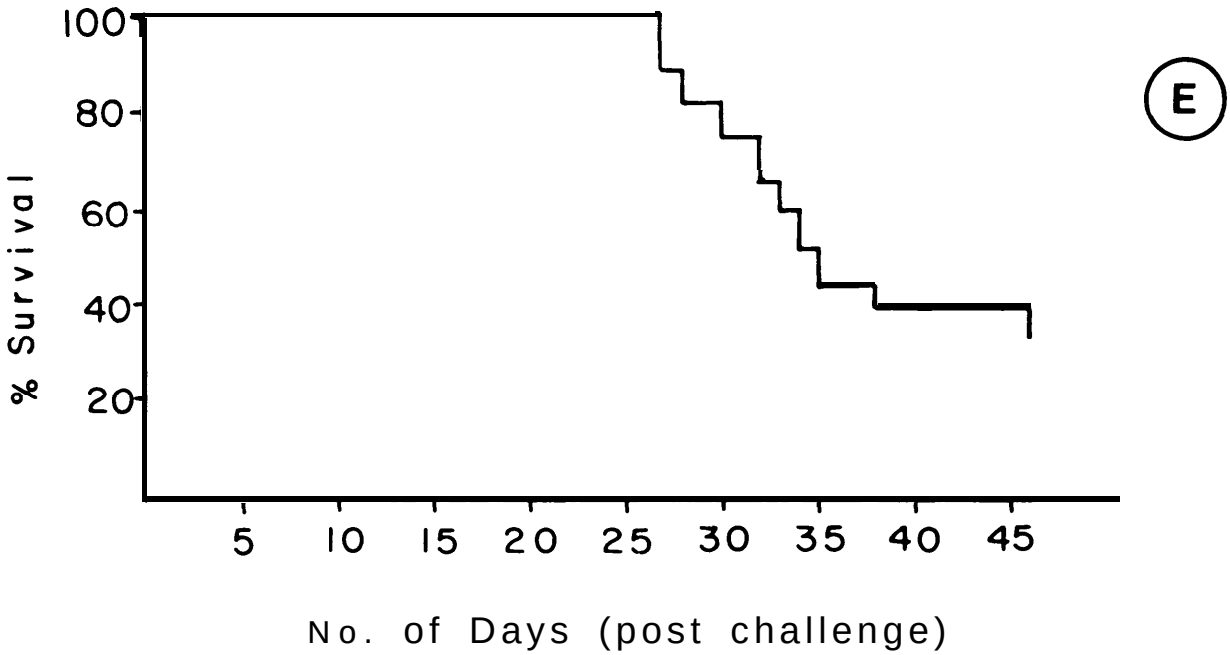


Figure 5. Cumulative mortalities - admixture experiment. Thirty animals were injected with 0.1 ml of a 1.0 O.D. KDB suspension. C = animals immunized with KD cells in the presence of E. coli lipopolysaccharide. D =KD cells in the presence of Vibrio anguillarum extract.



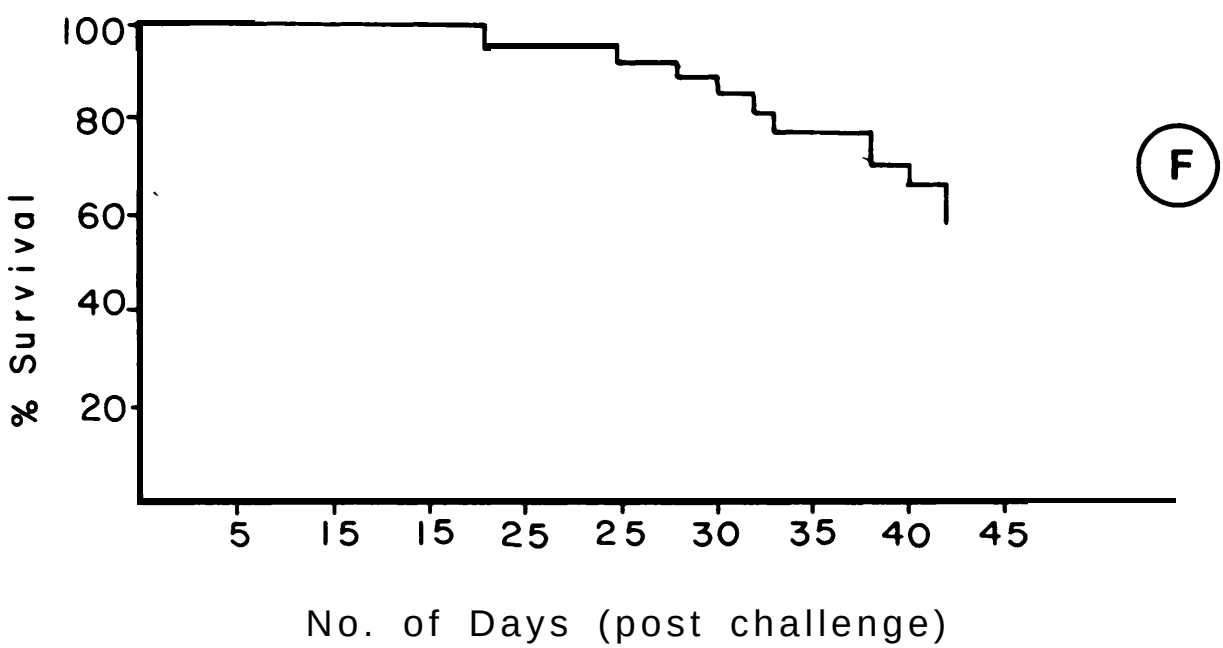


Figure 6. Cumulative mortalities -- admixture experiment. Thirty animals were injected with 0.1 ml of a 1.0 O.D. KDB suspension. E = animals immunized with KD cells emulsified in Freund's Incomplete adjuvant. F = Kd cells in PBS.

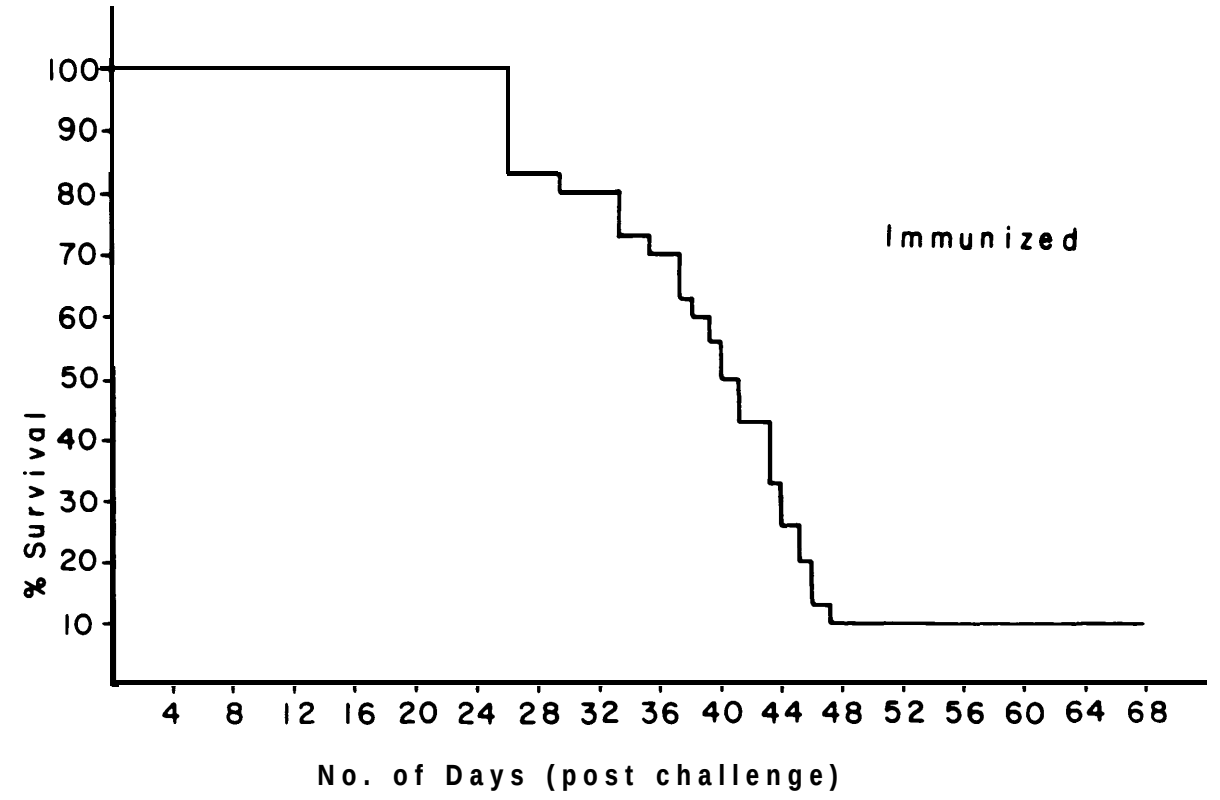
the end of the experiment (day 45) than any of the experimental groups, however, this group of animals were acclimitized for a different period of time thus the lower mortality is not significant.

This experiment points to a major problem with most KD challenge studies. Protection from KD may extremely difficult to demonstrate when the challenge procedure involves the intraperitoneal injection of a tubid suspension of the pathogen. Even animals which could be immune to a natural challenge may be overwhelmed by such a large antigenic load. To rectify this situaion we have developed an immersion challenge for coho (Results below), which we feel may more accurately reflect a natural challenge.

Soluble antigen-Vibrio cell conjugates. Due to the fact that formalin treated Vibrio anguillarum is such an effective vaccine for the prevention of vibriosis, it was reasoned that the covalent linkage of KDB antigens to the Vibrio cell surface could stimulate a protective immune response to kidney disease. In this manner it is anticipated that the KD antigen will appear in vivo as simply additional vibrio antigen.

Kidney disease antigens were covalently linked to the formalinized Vibrio cell surface by glutaraldehyde conjugation. Successful conjugation was ascertained by bacterial agglutination, using either rabbit anti-vibrio or anti-KD soluble antigen. Bacterial agglutination with rabbit anti-KD serum demonstrated a titer of l/1024 which was equivalent to the titer seen when the antiserum is tested directly against KD cells. Normal rabbit serum gave a titer of only l/16. Furthermore when the conjugate was tested against rabbit anti-vibrio serum. The titer was greater than l/2048. Thus the conjugate did not noticeably destroy much of the antigenicity of the Vibrio cells.

The results of the mortality study were positive, but did not demonstrate great protection (Fig 7). As can be seen from the figure all control animals



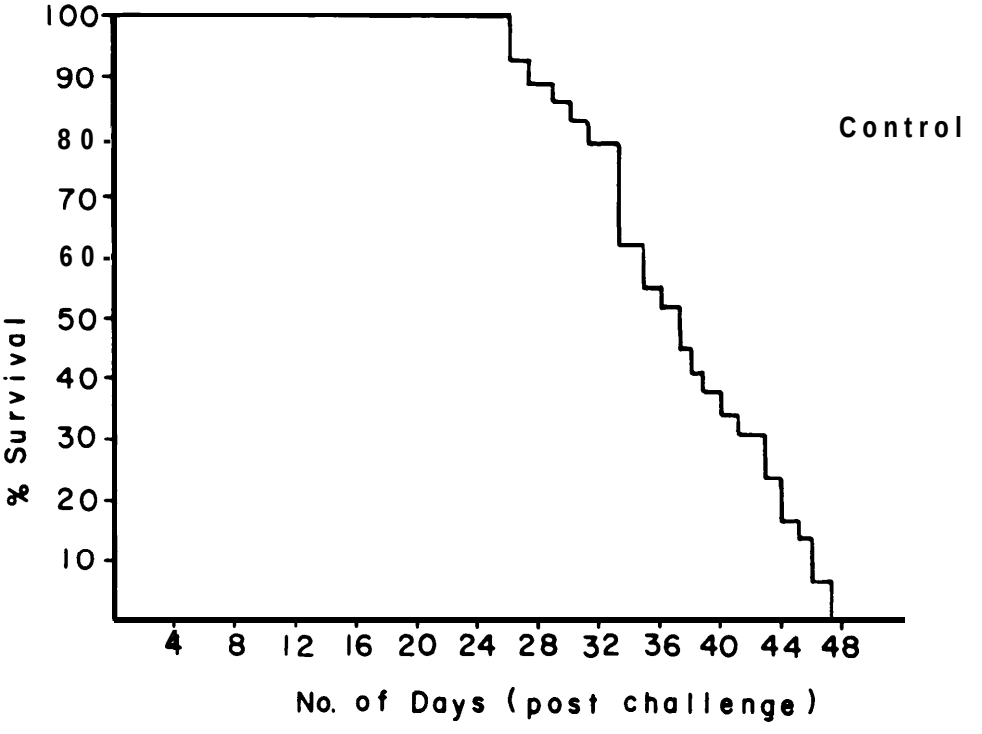


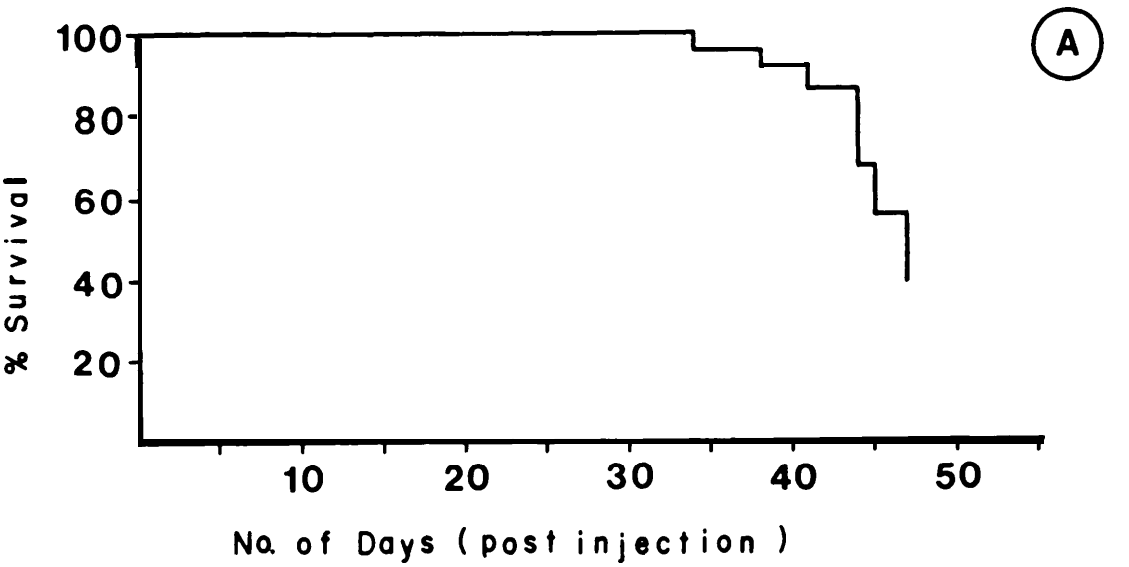
Figure 7. Cumulative mortalities - soluble antigen-Vibrio cell conjugate. Each group consisted of thirty individuals. The upper graph represents the animals immunized with the soluble antigen-Vibrio conjugate. The lower graph represents the control animals.

were dead by day 47, however 10% of the immunized animals survived until the termination of the experiment on day 68. Although the protection is not dramatic, we will be testing similar conjugates on coho using the immersion challenge procedure, which we feel to be much more sensitive.

The effect of live KD cell challenge preparations on mortality.

In an attempt to standardize all KD challenges the mortalities generated from different preparations of live KD were examined. This was undertaken, since, in our laboratory, using the same strain of KDB we could produce different mortality curves. Also, we wished to determine whether the simultaneous injection of other extrinsic or intrinsic factors could alter the mortality rate. Basically, few preparations of live KDB were prepared for challenge. All samples were produced from one culture (Fig. 8,9). They were all grown in 10% fetal calf serum in KDM-2. After sufficient growth, the organisms washed and resuspended in A) fresh KDM-2 plus serum with an additional 3.2 mg/ml KD soluble antigen, B) washed and resuspended in fresh KDM-2 with serum, C) washed and resuspended in fresh KDM-2 without serum, and D) washed and resuspended in the original supernatant. This experiment, then, was designed to test whether the presence or absence of fetal calf serum or soluble antigen produced during culture could be responsible for the fluctuations in mortality. From figures 8 and 9, it can be seen that the greatest mortality is generated by injecting the organism in its supernatant. All other groups gave lower degrees of mortality. In all subsequent intraperitoneal challenges, live cells are injected in their own growth medium.

Conjugation of Vibrio extract to KD cells. Vibrio extract which generates an excellent anti vibrio response (Kaattari and Irwin, 1985) was conjugated to formalin fixed R. salmoninarum cells. If the Vibrio extract contains some non-specific immunostimulatory maerial this form of vaccine



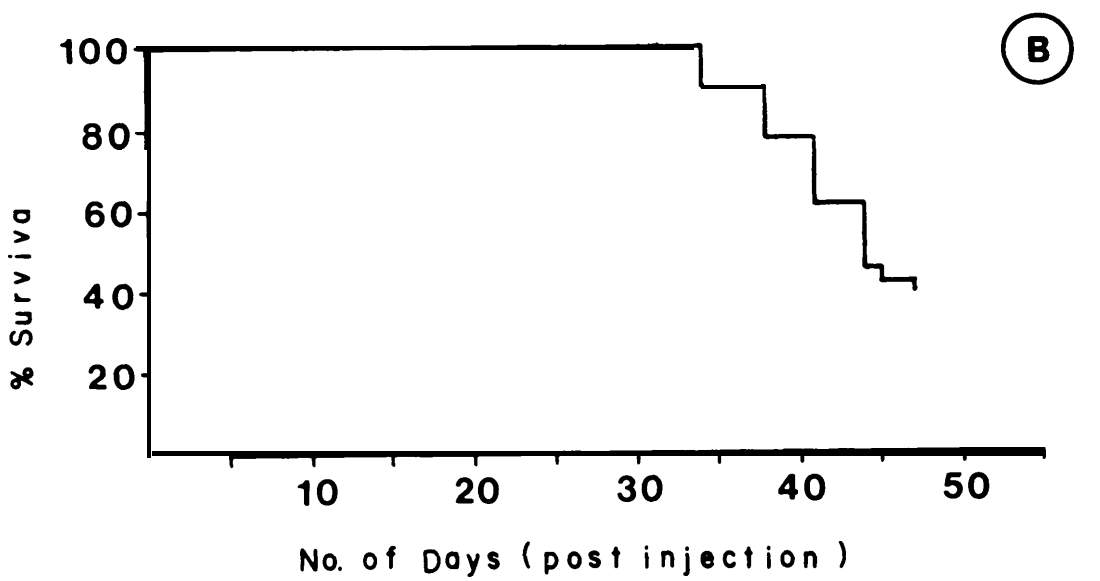
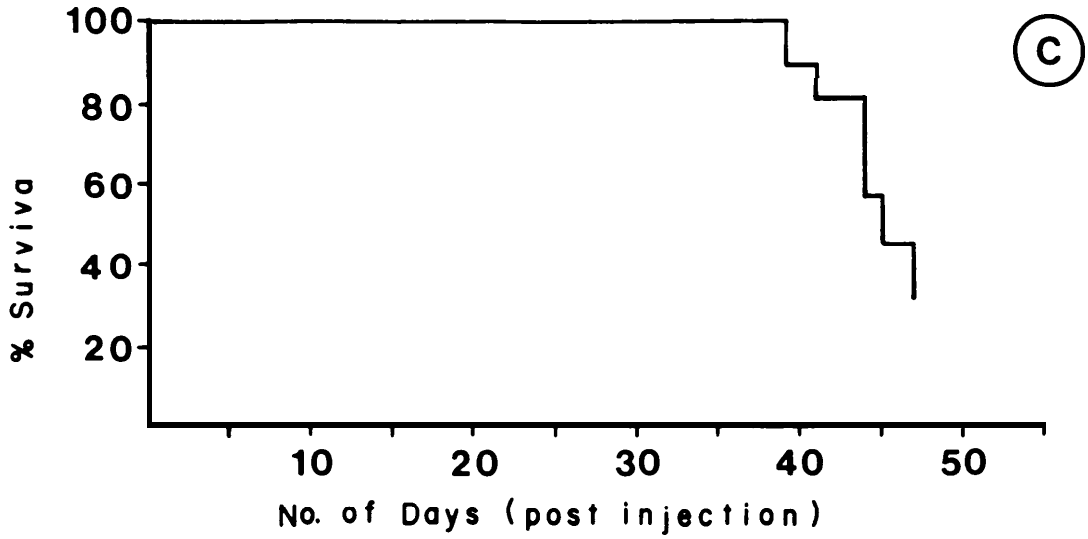


Figure 8. Effects of intrinsic and extrinsic factors on KD mortalities. A = KD cells resuspended in KDM-2 + 100x soluble antigen. B = KD cells resuspended in fresh KDM-2 with serum.



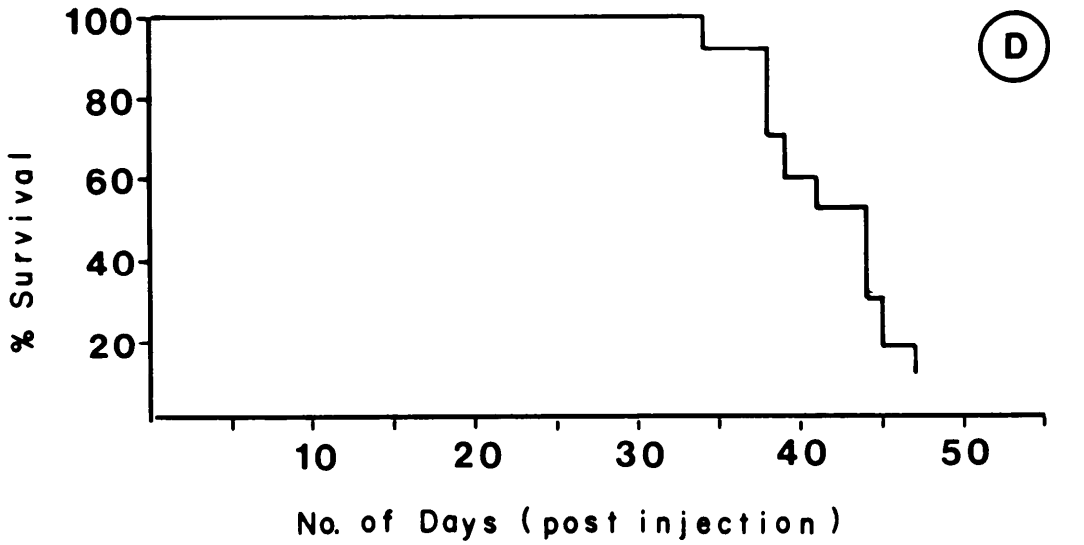


Figure 9. Effects of intrinsic and extrinsic factors on KD mortalities. C = KD cells resuspended in KDM-2 without serum, D = KD cells resuspended in the original supernatant.

could stimulate a response to adjacent R. salmoninarum determinants. This procedure made use of the procedure of Gray (1979), which differs from the cyanuric chloride procedure of Scibienski et al (1977), see below. The primary shortcomingfor this procedure is that it produces large aggregates of cells, instead of a single cell suspension. Such complexes would hamper the quantitation of the immunogen in the vaccine trials. Alternate routes of this form of conjugate were, then attempted to determine if any route would produce a conjugate that would remain in suspension.

Conjugation of Vibrio antigen extract to KD cells (method of Scibienski et al). This method of conjugation produced cells that remained in suspension. However, little vibrio antigen was detected on the KD cells as could be descerned by agglutination methods.

At this point it was decided some basic biochemical characterization of the Vibrio antigen extract should be done. Thus far, this has been limited to the fractionation of the antigen extract by differential ammonium sulfate precipitation. Each fraction was then assayed for protein content, and as further work is done, also analysis of carbohydrate and biological activity could be done. In this way the active immunostimulatory material will be purified and its basic chemical form identified. This, then, will aid in designing the most appropriate means for conjugation. This distribution of protein material can be seen in Table 1. The bulk of the protein can be differentially separated by saturated ammonium sulphate percentages from 20-42%.

Immersion challenge. As stated previously, one major drawback to the study of R. salmoninarum vaccine development, has been the inability to produce an appropriate method of challenge. The most successful method, thus far, has been the intraperitoneal injection of a high concentration of live KDB. This method insures the death of most of the challenged animals by day 60-90.

Table 1. Protein analysis of the differential saturated ammonium sulfate (SAS) precipitation of *Vibrio anguillarum* extracts.

Total SAS Added	Total Volume	% SAS	Protein Precipitated	% of Total protein
0.2 ml	2.2 ml	9	6.25 ug	0.4
0.5	2.5	20	6.25	0.4
1.0	3.0	33	656.25	41.3
1.5	3.5	42	566.25	35.7
2.0	4.0	50	120.00	7.5
8.0	10.0	80	231.00	14.5

* Initial volume of *V anguillarum* extract = 2 ml

The major problem with this method of challenge it may have been far too high a concentration, given in too vunerable a manner for even an immune animal to resist. Recently, repeated isolations of a KDB from dying salmon have produced a strain which can be introduced to salmon by the water-borne route and induce up 50% mortalities within 60 days. Armed with this method of challenge, we may have a more sensitive measure of the immunoprophylactic efficacy of our vaccines.

Assessments of immunity to kidney disease in salmon. Proper assessments of the immune status of the salmon immunized with various conjugates requires the development of various immunologic assays. In the final analysis, survival is the most important factor, however, with sensitive immunologic probes we can determine what form of immunity is most effective and which vaccines have the greatest potential for future development.

Therefore, we have developed three distinct means of evaluating the immune ressponse elicited by our vaccines to bacterial kidney disease. They are: 1) the detection of serum antibody to KD soluble antigen in salmon, 2) the detection of plaque forming cells to soluble antigen, and 3) the detection of antigen specific lymphocytic proliferation to KD.

Coho antibody responses to KD antigen. Figure 10 demonstrates that sera from coho salmon that were hyperimmunized to KD soluble antigen by repeated injections of antigen in Freund's complete adjuvant (left and center) produce good titers of antibody compared to the normal control (right).

Plaque-forming cell response to KD soluble antigen. Lymphocytes exposed in cell culture demonstated the capacity to generate plaque-forming cells that can lyse sheep red blood cells that are coated with soluble KD antigen (fig 11). It is of insterest that under these in vitro conditions we find that salmon lymphocytes are quite capable of generating

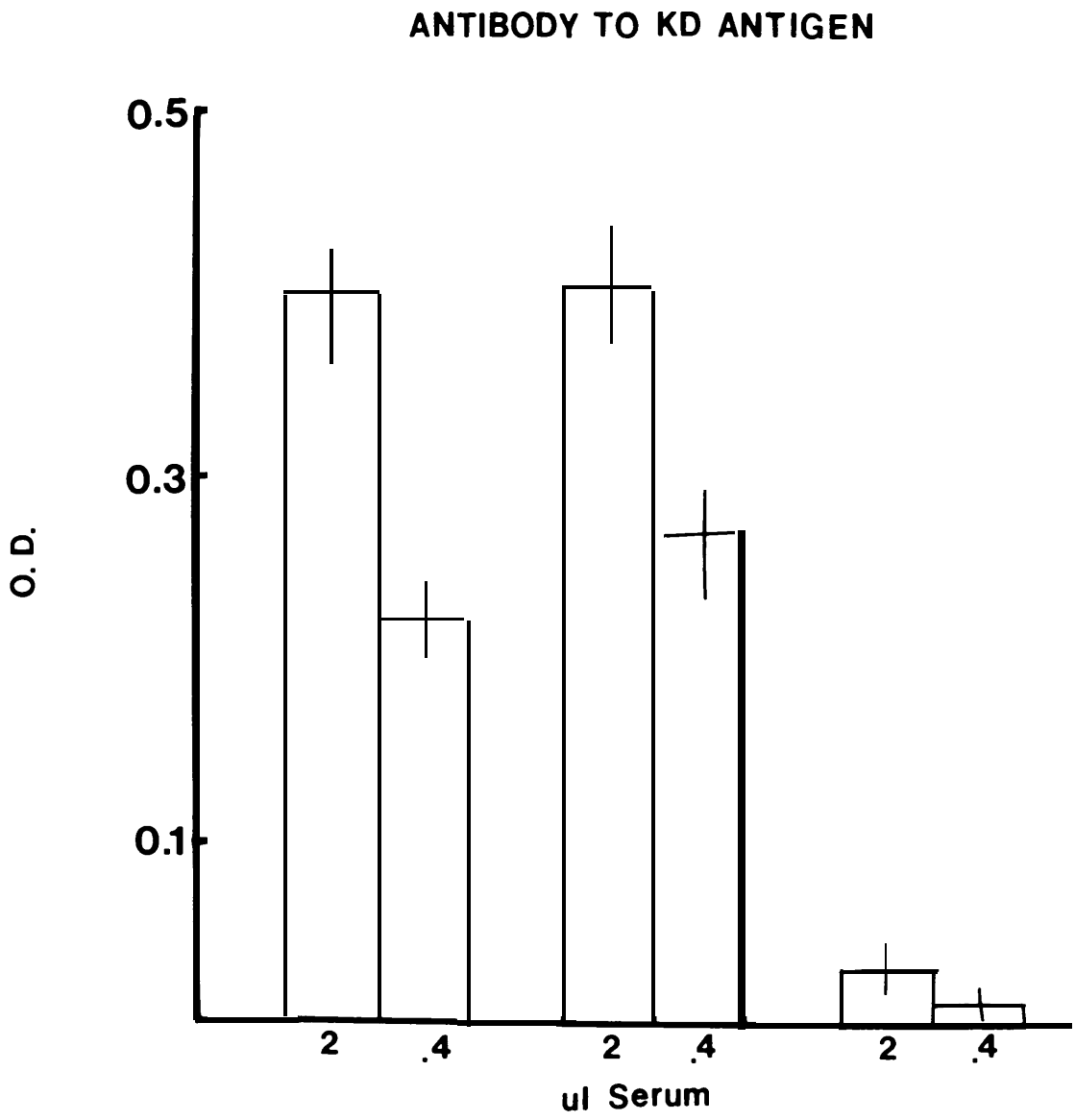


Figure 10. Coho anti-KD response as measured by ELISA. The first two pairs of dilutions of serum are taken from coho salmon immunized with KD cells in Freund's Complete adjuvant. The right pair represents the dilution of normal coho serum. The bars represent two standard errors about the mean of triplicates.

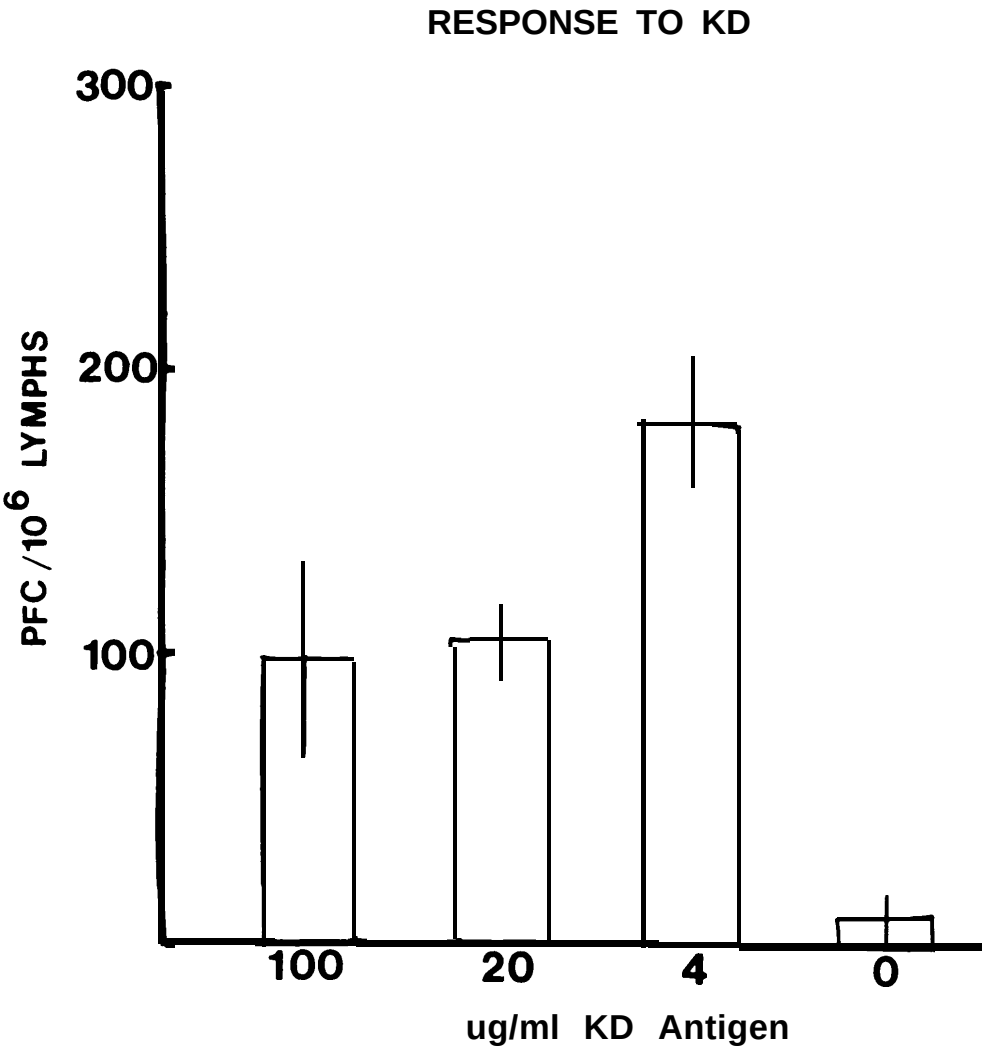


Figure 11. Coho plaque-forming response to KD soluble antigen. Coho anterior kidney lymphocytes were cultured for 9 days in the presence of various concentrations of soluble antigen. The PFC response is shown the mean of triplicate samples. The error bars represent two standard errors about the mean.

anti-KD producing cells. Perhaps this indicates that in vitro culture releases the salmonid lymphocytes from some negative regulatory control that normally prevents antibody production.

Cellular proliferation to KD soluble antigen. As shown in figure 12, animals which become immune, produce more antigen sensitive lymphocytes (black circles),and when subsequently exposed in vitro to the antigen they give a large proliferative response, as depicted on the right of the figure. This can be seen to occur when the lymphocytes from the various admixtures were examined (fig 3).

Identification of pathologically important antigens. The identification of toxins, adherence factors, and extracellular enzymes are important for two main reasons: 1) they often make the best candidates for vaccines, and 2) they provide clues as to the pathogenic mechanisms occurring during a disease. In the case of kidney disease, very little has been discoverd about the possible mechanisms of pathogenesis. However, recently Dr. A. Munro, Aberdeen, U.K. has found some association between the elaboration of a red cell binding antigen and the decreased hematocrits in KD infected animals(personal communication). We, also, have observed some of the KD soluble antigen binding red blood cells. Studies in this laboratory have shown that both salmon and rabbit red blood cells are capable of adsorbing a number of soluble antigens (fig. 13). Figure 13 is a photograph of a western blot showing that preincubation of whole soluble antigen (SA) with repeatedly with salmon (Fl-F4 or with rabbit red blood cells (Rl-R4) removes at least eight of the antigens recognize by the anti-KD-enzyme conjugate.

The possible existence of a hemolysin in the soluble antigen, which in turn may be responsible for an anemic state was examined by comparing

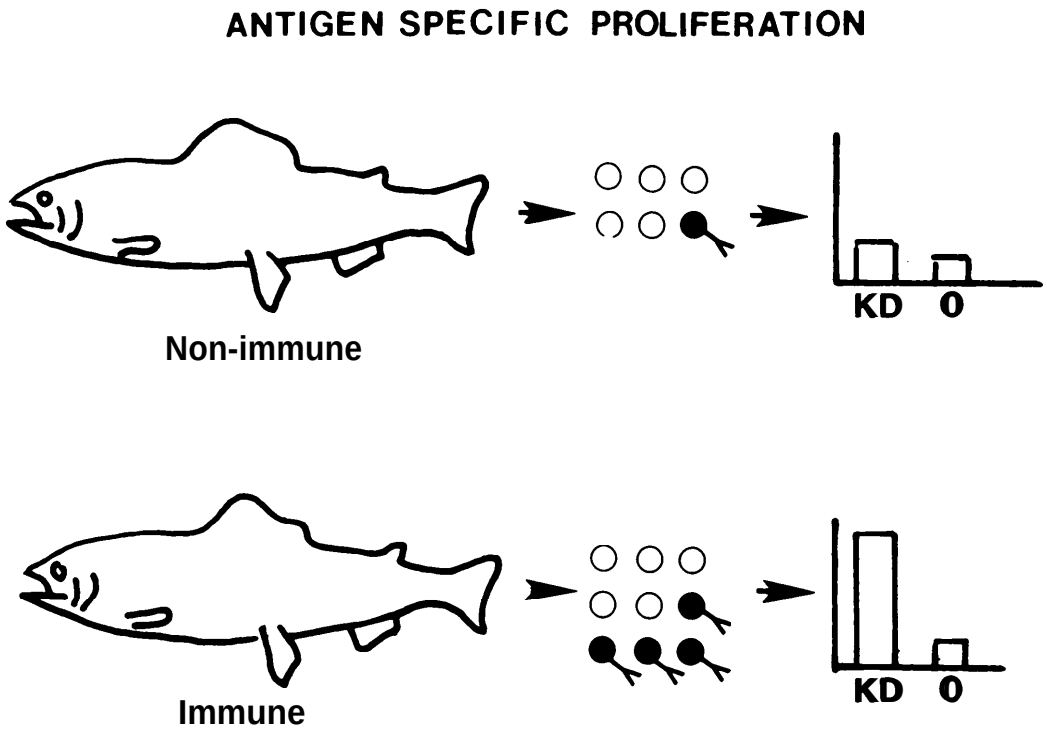


Figure 12. Graphical representation of the events occurring during antigen specific, cellular proliferation.

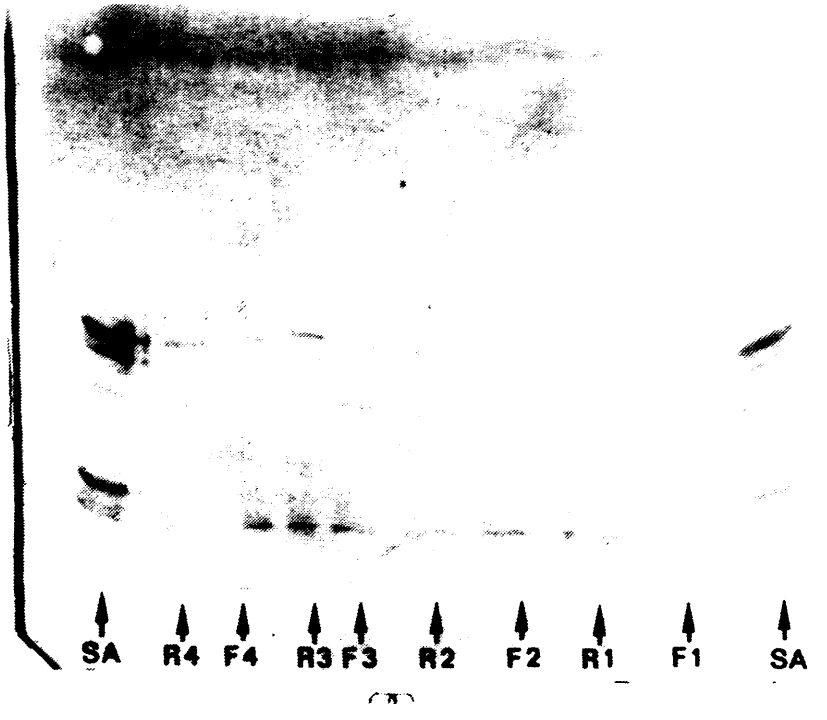


Figure 13. Western blot analysis, demonstrating protein antigens adsorbed specifically by salmon red blood cells (F1 - F4) or rabbit red blood cells (R1-R4). SA = unadsorbed soluble antigen. Adsorbed and unadsorbed antigens were electrophoresed on a PAGE gel, then transblotted onto nitrocellulose, where an antibody-conjugate and substrate was subsequently added.

hematocrits (% red blood cells in the blood) with soluble antigen levels in the serum of 19 artificially infected salmon. As can be seen from figure 14, high hematocrit values (28-45) correspond with low soluble antigen levels ($\leq$1 ug/ml) and low hematocrit values (5-23) correspond with high soluble antigen levels (100-1000 ug/ml). Biochemical characterization and isolation of this hemolytic activity is currently underway.

Immunosuppressive function of KD soluble antigen. Coincubation of physiological concentrations of soluble antigen (10-100 ug/ml) exert immunosuppressive action on the *in vitro* antibody response to TNP-lipopolysaccharide (fig 15). As can be seen from this figure, 100 ug/ml (C) and 10 ug/ml (D) suppress the normal antibody response (B), whereas control protein (ovalbumin) does not (F,G,H). Thus, this antigen may be giving a great advantage to the pathogen by destroying the salmon's immune defense system.

hematocrits (% red blood cells in the blood) with soluble antigen levels in the serum of 19 artificially infected salmon. As can be seen from figure 14, high hematocrit values (28-45) correspond with low soluble antigen levels ($\leq$1 ug/ml) and low hematocrit values (5-23) correspond with high soluble antigen levels (100-1000 ug/ml). Biochemical characterization and isolation of this hemolytic activity is currently underway.

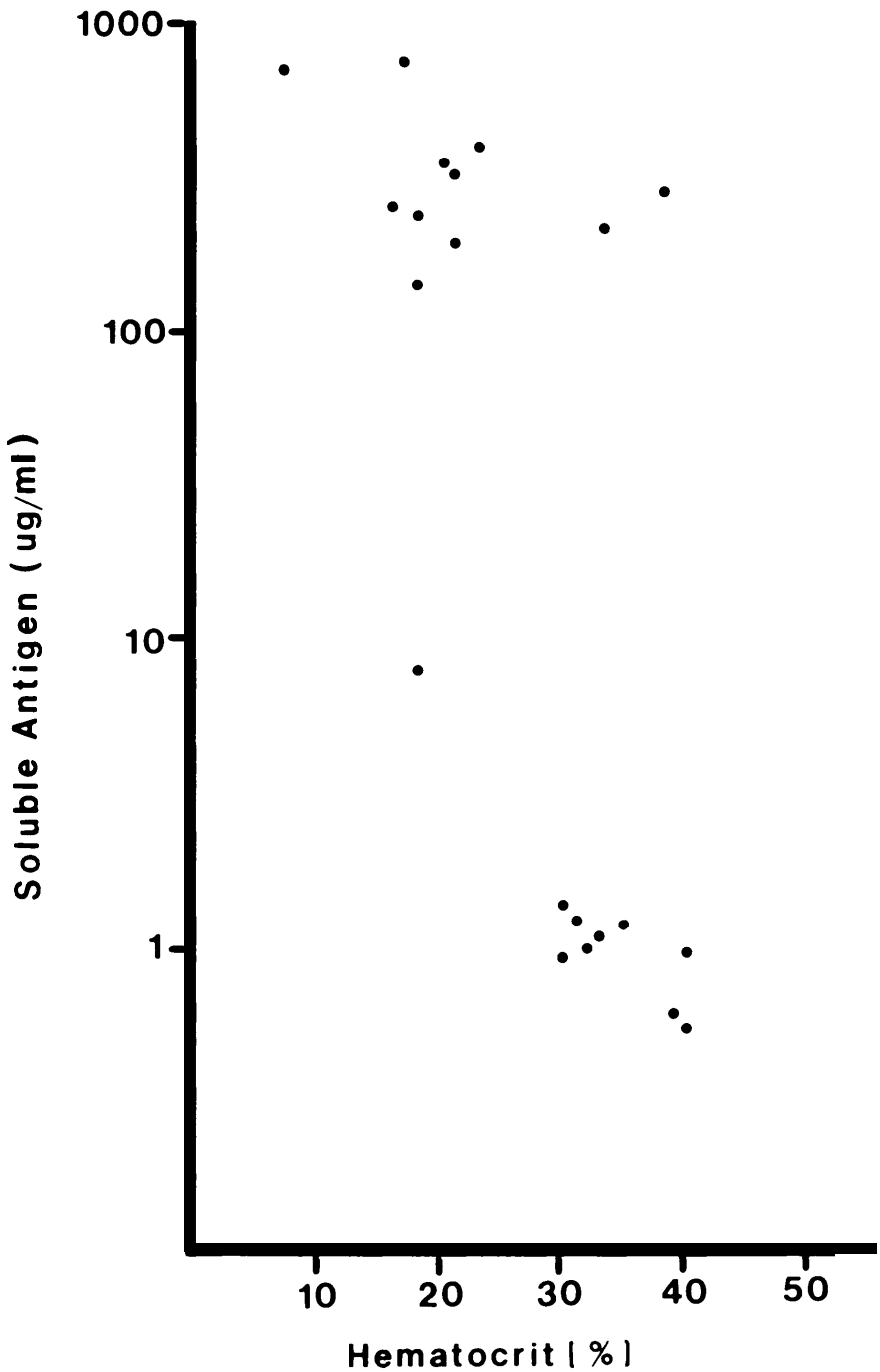


Figure 14. Correlation of soluble antigen level and hematocrit. Coho salmon were injected with 0.1 ml of 1.0 KDB. Fish were bled at 25 days. Each dot represents the hematocrit and soluble antigen level for one individual.

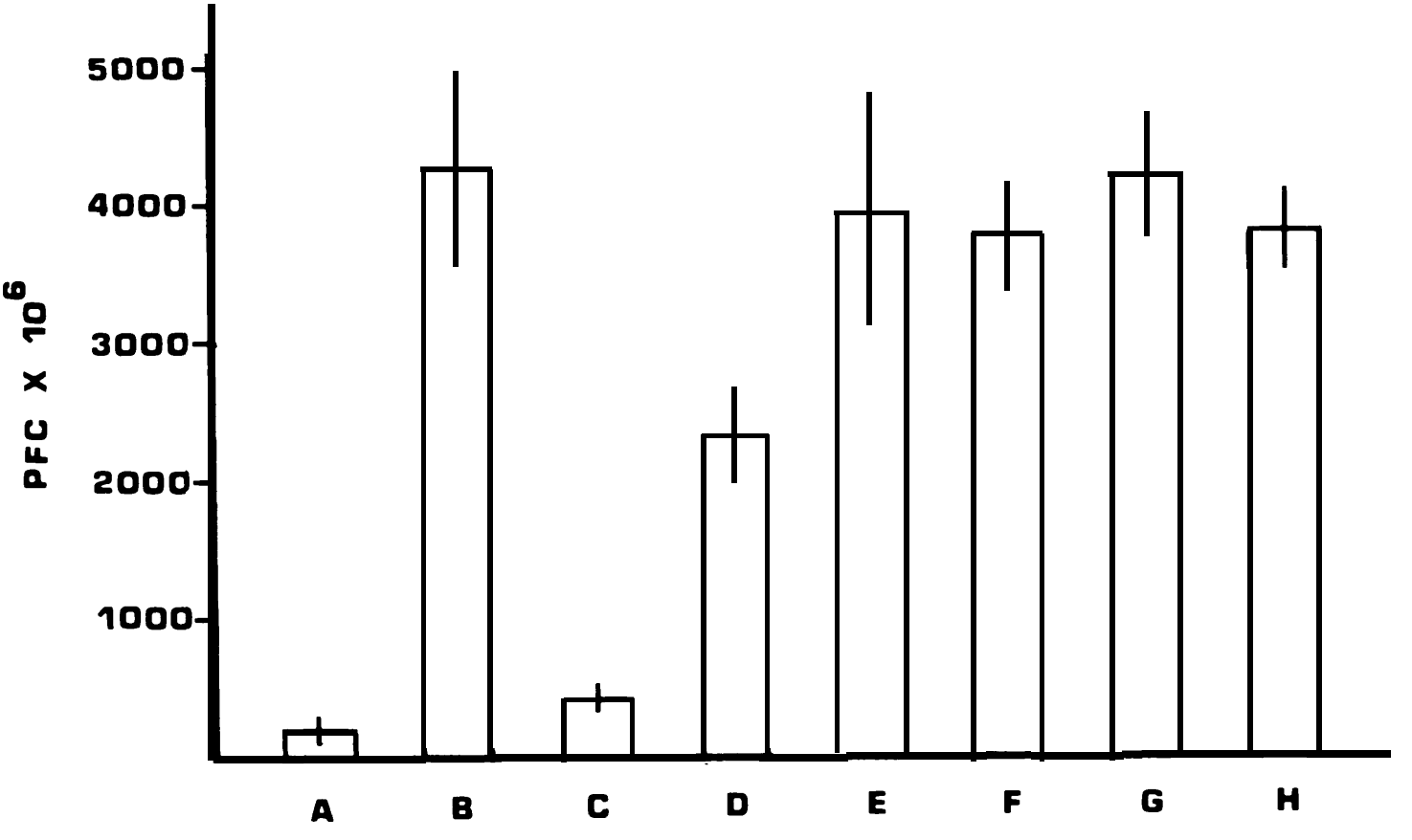


Figure 15. Immunosuppressive effects of soluble antigen. A = 0 antigen, B = TNP-LPS (4 ug/ml), C = TNP-LPS + 100 ug/ml soluble antigen (SA), D = TNP-LPS + 10 ug/ml SA, E = TNP-LPS + 1 ug/ml SA, F = TNP-LPS + 100 ug/ml ovalbumin (OVA), G = TNP-LPS + 10 ug/ml OVA, H = TNP-LPS + 1 ug/ml OVA.

SUMMARY AND CONCLUSIONS

The work of this second year has demonstrated may important facts and produced a number of vital procedures that will aid in the development of a vaccine for bacterial kidney disease.

I. Development of sensitive methods for assessing immunity to bacterial kidney disease. These include:

a. A method for immersion challenge of coho salmon
b. ELISA method for the detection of salmon anti-KD antibodies
c. A quantitative ELISA for the assessment of soluble antigen in the serum
d. A western blot assay for the diagnosis of infected fish

II. Assessment of techniques for the direct conjugation of antigen and immunostimulatory agents to bacterial cells.

III. Identification of toxins and antigens produced by R salmoninarum which may play an important role in pathogenesis, and, thus, may point to the best candidates for vaccine production.

SUMMARY OF EXPENDITURES

1.	Salaries (including personnel and benefits)	$ 45,509.98[a]
2.	Travel and transportation (including per diem)	876.46
3.	Nonexpendable equipment and material (greater than $1000 per item)	0.00
4.	Expendable equipment and material (sensitive in nature)	0.00
5.	Operations and maintenance (including computer services and publications)	19,527.09
6.	Overhead	20,721.49
7.	The currently approved budget	201,274.00
8.	Current budget period	7/l/85-6/31/86
9.	Cumulative expenses to date	157,107.36

a. Through 5/16/86

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